Evidence for a $\text{Na}^+/\text{Ca}^{2+}$ exchanger in neuroblastoma $\times$ glioma hybrid NG108-15 cells

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To determine whether NG108-15 cells contain a functional $\text{Na}^+/\text{Ca}^{2+}$ exchanger, we isotonicly replaced extracellular Na^+ with *N*-methyl-D-glucamine (NMG) and measured the effect on cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) using the fluorescent Ca^{2+} indicator fura 2. Replacement with NMG alone had no effect on basal $[\text{Ca}^{2+}]_i$ or the rise in $[\text{Ca}^{2+}]_i$ evoked by 80 mM K^+ or 10 μM bradykinin, but caused a larger $[\text{Ca}^{2+}]_i$ increase when thapsigargin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were added to the cells; this enhanced $[\text{Ca}^{2+}]_i$ increase

could be reversed by adding Na^+ back to the bathing buffer. The elevation in $[\text{Ca}^{2+}]_i$ induced by thapsigargin and FCCP was inversely proportional to extracellular Na^+ concentration. Furthermore, the exchanger operated in the reverse mode, as measured by either $[\text{Ca}^{2+}]_i$ change or $^{45}\text{Ca}^{2+}$ uptake. An 810 bp cDNA fragment of the exchanger was amplified by PCR; it differed by a single amino acid residue from the corresponding segment of the rat brain $\text{Na}^+/\text{Ca}^{2+}$ exchanger. These data suggest that a functioning $\text{Na}^+/\text{Ca}^{2+}$ exchanger exists in NG108-15 cells.

INTRODUCTION

Ca^{2+} is an important cellular mediator in many biological signalling processes in nerve cells [1,2]. In order to act as a signal, the cytosolic Ca^{2+} level ($[\text{Ca}^{2+}]_i$) must be able to undergo rapid and precisely controlled increase and return to the original low resting level. A relatively small increment in this level is frequently sufficient to trigger various Ca^{2+} -dependent physiological responses. When cells are excited electrically, activation of voltage-sensitive Ca^{2+} channels is predominantly responsible for Ca^{2+} influx [3]. On the other hand, release of intracellular sequestered Ca^{2+} induced by inositol 1,4,5-trisphosphate (IP_3) probably represents the major Ca^{2+} signal after receptor activation [4]. Recently, many reports have shown that extracellular Ca^{2+} influx is induced after agonist stimulation to replenish the intracellular Ca^{2+} pool and to prolong the Ca^{2+} signal within the cytosol [5,6].

Cells have evolved distinct mechanisms to remove accumulated Ca^{2+} from the cytosol in order to return to the resting state after activation [7,8]. At least three organelles contribute to $[\text{Ca}^{2+}]_i$ control in nerve cells, namely the plasma membrane, endoplasmic reticulum and mitochondria. Although Ca^{2+} can be sequestered into intracellular organelles, eventually a net Ca^{2+} efflux from cells is essential to lower the Ca^{2+} content after continuous extracellular Ca^{2+} influx in response to electrical excitation. There are two major transport mechanisms for extruding Ca^{2+} across the plasma membrane: a high-affinity Ca^{2+} pump which directly hydrolyses ATP to translocate Ca^{2+} ions out of cells [9], and a specific $\text{Na}^+/\text{Ca}^{2+}$ exchanger which utilizes the Na^+ gradient across the plasma membrane to drive extrusion of Ca^{2+} from the cytosol [10,11].

The physiological role of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in nerve cells is paradoxical; its capacity to transport Ca^{2+} is high, but it has only limited influence on $[\text{Ca}^{2+}]_i$ because of its low affinity for Ca^{2+} . It has been proposed that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger may

directly affect the amount of Ca^{2+} stored in intracellular organelles and consequently may regulate the size of IP_3 -sensitive Ca^{2+} pools [12]. The neuroblastoma $\times$ glioma hybrid NG108-15 cell line is a useful model system for studying many aspects of neuronal signalling pathways. In the present study, we found that basal $[\text{Ca}^{2+}]_i$ and the transient of $[\text{Ca}^{2+}]_i$ evoked by either high K^+ or bradykinin were independent of extracellular Na^+ in NG108-15 cells. However, when Ca^{2+} sequestration within intracellular organelles was blocked by thapsigargin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger contributed to the regulation of $[\text{Ca}^{2+}]_i$.

MATERIALS AND METHODS

Culture of neuroblastoma $\times$ glioma hybrid NG108-15 cells

NG108-15 cells obtained from Dr. M. Nirenberg (National Institutes of Health, Bethesda, MD, U.S.A.) were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, U.S.A.) supplemented with 100 μM hypoxanthine, 1 μM aminopterin, 16 μM thymidine (Gibco-BRL) and 10% fetal bovine serum (Gibco-BRL). The cells were used between passages 21 and 40 and were grown at 37 °C in 100 mm $\times$ 20 mm tissue-culture dishes (Falcon) in a humidified atmosphere of 10% CO_2 /90% air. Differentiation was induced by incorporation of 1 mM dibutyryl cyclic AMP into the growth medium and reduction of serum to 5% for a period of 5 days. The medium was changed daily by replacing half of the volume of growth medium with fresh medium.

Cytosolic Ca^{2+} measurement

NG108-15 cells ($1 \times 10^6/\text{ml}$) were harvested in standard buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 2.2 mM CaCl_2 , 5 mM

Abbreviations used: NMG, *N*-methyl-D-glucamine; IP_3 , inositol 1,4,5-trisphosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concentration.

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glucose and 10 mM Hepes, pH 7.4) and incubated with 5 μ M fura 2 acetoxymethyl ester at 37 °C for 30 min. After three washes with standard buffer, cells were suspended in the same buffer at 1×10^6 /ml and kept on ice before use. Aliquots of cells were centrifuged once before fluorescence was measured. The fluorescence emitted by cells (2×10^5) at 505 nm was measured in a cuvette at room temperature using a dual-wavelength spectrofluorimeter system (CM system, Spex Industries, Edison, NJ, U.S.A.) with excitation at 340 nm and 380 nm. $[Ca^{2+}]_i$ was calculated from the ratios of fluorescence intensities at 340 nm and 380 nm and a K_d of 135 nM as described previously [13]. The maximum fluorescence ratio was obtained by adding 0.01 % digitonin to the cuvette at the end of the experiment; the minimum fluorescence ratio was determined subsequently by adding 50 mM EGTA. For the experiments in which various extracellular Na^+ concentrations were substituted, the required NaCl in the standard buffer was isotonicity supplemented with *N*-methyl-D-glucamine (NMG). Some experiments were carried out in the absence of extracellular Na^+ in which NaCl in the standard buffer was totally isotonicity replaced with NMG (designated as Na^+ -free NMG buffer). When cells were depolarized by high K^+ , the extracellular K^+ concentration was raised by adding to the cells an equal volume of standard buffer in which the NaCl was totally isotonicity replaced with KCl. Osmolality was measured using a freezing-point osmometer (Advanced Instruments, Norwood, MA, U.S.A.). All experiments were performed at least three times using different batches of cells. Results from one representative experiments are illustrated graphically, and mean $\pm$ S.D. values from peak $[Ca^{2+}]_i$, calculated from n experiments, are shown.

Na^+ loading

The cytosolic Na^+ concentration was elevated in order to determine whether the Na^+/Ca^{2+} exchanger operated in the reverse mode. Ouabain at 100 μ M had been incorporated into the loading mixture during the last 15 min of the fura-2 incubation period. After being washed, cells were suspended in either Ca^{2+} -free Na^+ -containing standard buffer or Ca^{2+} -free Na^+ -free NMG buffer. Verapamil (100 μ M) and ω -conotoxin (10 μ M) were added to these cells for subsequent $[Ca^{2+}]_i$ measurement to prevent voltage-operated Ca^{2+} influx due to cytosolic Na^+ elevation.

Determination of $^{45}Ca^{2+}$ uptake

After cells (1×10^6 /ml) had been incubated with 100 μ M ouabain at 37 °C for 15 min in standard buffer, they were suspended in a Ca^{2+} -free Na^+ -free NMG buffer at a density of 5×10^6 /ml. Ca^{2+} uptake was initiated by adding 20 μ l aliquots of cells (about 0.4 mg of cell protein) to test tubes containing 200 μ l of various concentrations of Na^+ -substitute standard buffers with 100 μ M $^{45}Ca^{2+}/CaCl_2$ (8×10^6 c.p.m./ml), 100 μ M verapamil and 10 μ M ω -conotoxin. At the indicated times, $^{45}Ca^{2+}$ uptake was terminated by the addition of 4 ml of ice-cold standard buffer containing 2 mM $LaCl_3$, followed by immediate vacuum filtration of the cells on to Whatman GF/C glass-fibre filters. After another four washes, the radioactivity remaining on the filters was counted, and the amount of Ca^{2+} that had accumulated within the cells was thereby deduced. A control experiment without ouabain pretreatment was also included. For experiments designed to measure the Ca^{2+} -dependence of $^{45}Ca^{2+}$ uptake, Na^+ -free NMG buffers containing various concentrations of Ca^{2+} were made up with the same specific radioactivity of $^{45}Ca^{2+}/CaCl_2$ (0.6 μ Ci/mol).

cDNA preparation and PCR

After aspiration of the growth medium, cells were washed three times with standard buffer. Isolation of total RNA was carried out using an RNazol B kit from Biotecx Laboratories (Houston, TX, U.S.A.). The first-strand cDNA was then synthesized by SuperScript MuMLV reverse transcriptase from Gibco-BRL using total RNA as the template. On the basis of the nucleotide sequences of both canine and human heart Na^+/Ca^{2+} exchangers [14,15], two oligonucleotides located in the largest intracellular loop between the amino acids at positions 270 and 538 of the corresponding human heart Na^+/Ca^{2+} exchanger sequence were designed. These oligonucleotide primers were used for PCR to amplify the cDNA fragment isolated from NG108-15 cells. The sequences of the two primers were: 5' sense primer, CTTAGATGGTGCTCTGGTTCTGGAGG; and 3' anti-sense primer, GGAACGATAACATTTCTCGAGCTCC. PCR was performed for 30 cycles (94 °C, 0.5 min; 50 °C, 1 min; and 72 °C, 2 min) as recommended by the manufacturer (Perkin-Elmer Cetus, Norwalk, CT, U.S.A.). The predicted size of the PCR product was 810 bp, and products were analysed by electrophoresis on a 0.8 % agarose gel.

Subcloning and DNA sequencing

The amplified cDNA fragment was ligated with pGEM-T vector (Promega, Madison, WI, U.S.A.) after extraction from the agarose gel using a JETsorb kit from Genomed (Bad Oeynhausen, Germany). After being subcloned in competent JM109 *E. coli*, plasmids were purified using a Magic Minipreps DNA Purification System kit from Promega. The cDNA fragment was finally sequenced using a Sequenase Version 2.0 DNA Sequencing kit from United States Biochemical (Cleveland, OH, U.S.A.).

Materials and miscellaneous procedures

The fluorescent Ca^{2+} indicator fura 2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR, U.S.A.). Bradykinin, ouabain, dibutyryl cyclic AMP, NMG, verapamil, FCCP and ω -conotoxin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Thapsigargin was provided by Research Biochemicals (Natick, MA, U.S.A.). $^{45}Ca^{2+}$ (35.12 mCi/mg) was bought from New England Nuclear (Boston, MA, U.S.A.).

Protein concentrations were determined by the method of Lowry et al. [16] using BSA standards. All experiments for measurement of $^{45}Ca^{2+}$ uptake were carried out at least three times in triplicate with similar results, and the data presented are means $\pm$ S.D. from one representative triplicate experiment.

RESULTS

In an attempt to demonstrate the participation of the Na^+/Ca^{2+} exchanger in the mediation of Ca^{2+} signalling in NG108-15 cells, we measured the change in $[Ca^{2+}]_i$ in response to high K^+ in the presence of various extracellular Na^+ concentrations. Extracellular Na^+ in the standard buffer was replaced with NMG isotonicity as needed. To maintain normal tonicity, high K^+ (80 mM) was achieved by adding an equal volume of a buffer in which the NaCl in the standard buffer was totally replaced isotonicity with KCl. As shown in Figure 1(a), when cells were suspended in standard buffer containing 150 mM Na^+ , 80 mM K^+ induced a rapid increase in $[Ca^{2+}]_i$ from a basal level of 70 ± 15 nM ($n = 18$) to a mean peak of 288 ± 17 nM ($n = 9$). After reaching the peak, $[Ca^{2+}]_i$ gradually decreased up to 120 s.

Figure 1 Effect of extracellular Na⁺ on change in [Ca²⁺]_i stimulated by high K⁺ or bradykinin in NG108-15 cells

(a) Fura-2-loaded NG108-15 cells (2×10^5) were added to a cuvette containing 1.5 ml of Na⁺-containing standard buffer (trace A) or Na⁺-free NMG buffer (trace B). The extracellular K⁺ concentration was raised by adding an equal volume of standard buffer in which the NaCl was totally isotonicly replaced with KCl as indicated by the arrowhead to give a final concentration of 80 mM K⁺. (b) Bradykinin (BK) at 10 μ M was added as indicated (arrowhead) to fura-2-loaded NG108-15 cells suspended in the Na⁺-containing standard buffer (trace A) or Na⁺-free NMG buffer (trace B). (c) The basal level (●) and the peak level of [Ca²⁺]_i stimulated by high K⁺ (○) or bradykinin (▲), measured at various concentrations of extracellular Na⁺, are demonstrated. Data shown are means $\pm$ S.D. ($n = 9$).

[Ca²⁺]_i was 191 ± 18 nM ($n = 9$) 120 s after the addition of 80 mM K⁺ (Figure 1a, trace A). The increase in [Ca²⁺]_i induced by 80 mM K⁺ and its decline after reaching the peak displayed a similar profile even when cells were suspended in Na⁺-free NMG buffer in which extracellular Na⁺ was completely replaced with NMG (Figure 1a, trace B). The mean basal, peak and 120 s values of [Ca²⁺]_i after the addition of 80 mM K⁺ were 78 ± 12 ($n = 18$), 310 ± 10 ($n = 9$) and 202 ± 23 nM ($n = 9$), respectively. There was no clear difference in [Ca²⁺]_i transient stimulated by high K⁺ between cells suspended in Na⁺-containing standard buffer and Na⁺-free NMG buffer. To understand further the role of the Na⁺/Ca²⁺ exchanger in the regulation of [Ca²⁺]_i, the [Ca²⁺]_i of NG108-15 cells was elevated by using a different agonist, bradykinin. The results are illustrated in Figure 1(b). Bradykinin (10 μ M) caused a greater increase in [Ca²⁺]_i than did high K⁺. The mean peak levels were 386 ± 30 ($n = 9$) and 364 ± 22 nM ($n = 9$) in the presence and absence of extracellular Na⁺ respectively. In addition, the elevated [Ca²⁺]_i elicited by bradykinin

Figure 2 Thapsigargin-induced changes in [Ca²⁺]_i of NG108-15 cells in the presence or absence of extracellular Na⁺

Thapsigargin (TG) (1 μ M) was separately added, as indicated by the arrowhead, to fura-2-loaded NG108-15 cells suspended in Na⁺-containing standard buffer (trace A) or Na⁺-free NMG buffer (trace B).

declined to basal level within 15 s of the peak being reached (Figure 1b, traces A and B), and the rate of [Ca²⁺]_i decline was not affected by extracellular Na⁺. Figure 1(c) depicts the dependence of the changes in [Ca²⁺]_i elicited by high K⁺ or bradykinin on extracellular Na⁺ concentration. Resting and peak [Ca²⁺]_i induced by both agonists remained the same for all extracellular Na⁺ concentrations tested.

The results described above suggest that the Na⁺/Ca²⁺ exchanger may not have actively functioned in NG108-15 cells. However, the changes in [Ca²⁺]_i measured represent the final [Ca²⁺]_i after the operation of all Ca²⁺ transporters within the cell. If other Ca²⁺ transporters, e.g. thapsigargin-sensitive Ca²⁺-ATPase, the uniporter in mitochondria and the plasma-membrane Ca²⁺ pump, actively removed the accumulated cytosolic Ca²⁺, it would have been difficult to observe the operation of the Na⁺/Ca²⁺ exchanger by measurement of [Ca²⁺]_i in intact cells. To study further whether the Na⁺/Ca²⁺ exchanger in NG108-15 cells plays a functional role in Ca²⁺ regulation, we blocked the Na⁺-independent mechanisms that decrease [Ca²⁺]_i. First, cells were treated with 1 μ M thapsigargin to inhibit the Ca²⁺-pumping ATPase activity of internal stores [17]. Figure 2 shows that application of 1 μ M thapsigargin resulted in an increase in [Ca²⁺]_i, which reached a peak of 155 ± 28 nM ($n = 7$) in approx. 35 s (trace A). Removal of extracellular Na⁺ from another population of cells resulted in an increase in [Ca²⁺]_i to a peak of 247 ± 30 nM ($n = 7$) (trace B). The peak [Ca²⁺]_i induced by thapsigargin was 60% higher in cells exposed to Na⁺-free NMG buffer than in those exposed to Na⁺-containing standard buffer. Furthermore 120 s after the addition of thapsigargin [Ca²⁺]_i increased from 66 ± 16 nM ($n = 7$) in the presence of extracellular Na⁺ to 94 ± 21 nM ($n = 7$) in its absence.

The sensitivity of [Ca²⁺]_i changes to extracellular Na⁺ described above suggests that there is Na⁺/Ca²⁺ exchanger activity in NG108-15 cells. To study further whether the function of this exchanger is even more relevant in [Ca²⁺]_i regulation when other Ca²⁺-removal routes from the cytosol are blocked, a mitochondrial uncoupler, FCCP, was used together with thapsigargin. FCCP abolishes mitochondrial membrane potential, thereby

Figure 3 Dependence of $[Ca^{2+}]_i$ increase evoked by thapsigargin and FCCP on extracellular Na^+ concentration in NG108-15 cells

(a) Fura-2-loaded NG108-15 cells were added to cuvettes, as indicated by the arrowhead, that contained 1 μ M thapsigargin and 10 μ M FCCP and various concentrations of Na^+ (150 mM, trace A; 60 mM, trace B; 30 mM, trace C; and 0 mM, trace D). In each trace, the required Na^+ in the standard buffer was replaced isotonicly by NMG. (b) Maximal level of $[Ca^{2+}]_i$ induced by 1 μ M thapsigargin and 10 μ M FCCP in the presence of various concentrations of extracellular Na^+ as described in (a). Results are means $\pm$ S.D. ($n = 7$).

blocking mitochondrial Ca^{2+} uptake and inducing a loss of sequestered Ca^{2+} from the mitochondria. The effect of the removal of extracellular Na^+ on the thapsigargin- and FCCP-induced rise in $[Ca^{2+}]_i$ is shown in Figure 3. The $[Ca^{2+}]_i$ transient induced by thapsigargin (1 μ M) and FCCP (10 μ M) was greater in cells bathed in Na^+ -free NMG buffer than in those bathed in Na^+ -containing standard buffer; the $[Ca^{2+}]_i$ increase was 463 ± 44 nM ($n = 7$) in control cells (Figure 3a, trace A) compared with 800 ± 26 nM ($n = 7$) in cells exposed to Na^+ -free NMG buffer (Figure 3a, trace D). As the concentration of Na^+ increased, the magnitude of the rise in $[Ca^{2+}]_i$ induced by thapsigargin and FCCP decreased. Thus the mean peaks of $[Ca^{2+}]_i$ were 664 ± 38 nM ($n = 7$) (Figure 3a, trace C) and 498 ± 18 nM ($n = 7$) (Figure 3a, trace B) in the presence of 30 and 60 mM extracellular Na^+ respectively. Figure 3(b) demonstrates the dependence of the changes in $[Ca^{2+}]_i$ elicited by thapsigargin and FCCP on extracellular Na^+ concentration. Furthermore, the enhanced $[Ca^{2+}]_i$

Figure 4 Restoration of enhanced $[Ca^{2+}]_i$ increase induced by Na^+ replacement

Fura-2-loaded NG108-15 cells were suspended in Na^+ -containing standard buffer (trace A) or Na^+ -free NMG buffer (traces B–D) in a cuvette. Thapsigargin (TG) (1 μ M) and FCCP (10 μ M) were added to the cells as indicated by the arrow. At the time indicated by the arrowhead, 90 mM Na^+ (trace B) or 90 mM NMG (trace D) was added.

increase observed in cells bathed in the Na^+ -free NMG buffer was reversed by readdition of 90 mM Na^+ to the bathing buffer, and $[Ca^{2+}]_i$ rapidly returned to the same level as that in control cells bathed in Na^+ -containing standard buffer (Figure 4, trace B). To examine the effect of the change in the tonicity, the same concentration of NMG was added as a control. No significant effect on $[Ca^{2+}]_i$ was observed (Figure 4, trace D). This result indicates that the larger increase in $[Ca^{2+}]_i$ observed in cells exposed to Na^+ -free NMG buffer was specifically due to the removal of extracellular Na^+ .

The characteristics of the Na^+/Ca^{2+} exchanger activity in intact NG108-15 cells were further investigated by establishing an outwardly directed Na^+ gradient across the plasma membrane and then examining its effects on $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ uptake via the reverse mode of the exchanger. Cytosolic Na^+ level was increased by inhibiting the Na^+ pump with 100 μ M ouabain. Cells were then suspended in Ca^{2+} -free buffer either with or without extracellular Na^+ and containing 100 μ M verapamil and 10 μ M ω -conotoxin. When extracellular Ca^{2+} was added to cells, $[Ca^{2+}]_i$ rapidly rose in both sets of cells. However, there was a larger increase in $[Ca^{2+}]_i$ in cells bathed in Na^+ -free NMG buffer than in those bathed in Na^+ -containing standard buffer (Figure 5a). The mean peaks of $[Ca^{2+}]_i$ were 98 ± 10 nM ($n = 8$) and 66 ± 6 nM ($n = 8$) in cells exposed to Na^+ -free and Na^+ -containing buffer respectively. This increase in $[Ca^{2+}]_i$ observed in cells exposed to Na^+ -free NMG buffer was reversed by adding Na^+ back to the bathing buffer, and $[Ca^{2+}]_i$ eventually returned to the same level as in cells bathed in the Na^+ -containing standard buffer (Figure 5a). Under similar experimental conditions, after ouabain pretreatment, greater $^{45}Ca^{2+}$ uptake was observed in cells bathed in Na^+ -free NMG buffer than in those

Figure 5 Outwardly directed Na⁺-gradient-driven Ca²⁺ influx in NG108-15 cells

(a) After incubation with 100 μ M ouabain for 15 min, fura-2-loaded NG108-15 cells were suspended in Ca²⁺-free Na⁺-containing standard buffer (trace A) or Ca²⁺-free Na⁺-free NMG buffer (trace B) containing 100 μ M verapamil and 10 μ M ω -conotoxin. Ca²⁺ (1.1 mM) was added as indicated by the arrowhead. At the time indicated by the arrow (trace B only), 150 mM NaCl was added. (b) Ouabain-pretreated NG108-15 cells were suspended in Ca²⁺-free Na⁺-free NMG buffer. ⁴⁵Ca²⁺ uptake was initiated by adding cells to test tubes containing 100 μ M ⁴⁵Ca²⁺, 100 μ M verapamil and 10 μ M ω -conotoxin in the Na⁺-containing standard buffer (●) or Na⁺-free NMG buffer (○). ⁴⁵Ca²⁺ uptake was terminated at the indicated time. Data are means $\pm$ S.D. of one triplicate experiment.

bathed in Na⁺-containing standard buffer (Figure 5b). Thus the apparent initial rate of ⁴⁵Ca²⁺ influx and the maximum ⁴⁵Ca²⁺ uptake in cells bathed in Na⁺-free NMG buffer were 0.086 ± 0.001 pmol/s per mg and 3.2 ± 0.16 pmol/mg ($n = 3$) respectively as opposed to 0.063 ± 0.007 pmol/s per mg and 1.91 ± 0.08 pmol/mg ($n = 3$) in control cells bathed in Na⁺-containing standard buffer (Figure 5b).

⁴⁵Ca²⁺ influx induced by ouabain pretreatment was dependent on an outwardly directed Na⁺ gradient. Therefore when the Na⁺ concentration was gradually raised in the bathing buffer to minimize the Na⁺ gradient in ouabain-pretreated cells, the ⁴⁵Ca²⁺ uptake was proportionally reduced, as depicted in Figure 6. Figure 6 further indicates that the operation of the reverse mode of the Na⁺/Ca²⁺ exchanger activity was indeed dependent on an outwardly directed Na⁺ gradient. Without ouabain pretreatment, a gradient of Na⁺ concentration did not build up within cells, so ⁴⁵Ca²⁺ uptake remained at a constant level regardless of the concentration of Na⁺ in the bathing buffer. Figure 7 shows how the Na⁺-gradient-driven ⁴⁵Ca²⁺ uptake depends on extracellular Ca²⁺ concentration. The maximal effect occurred at Ca²⁺ concentrations greater than 1 mM and the apparent concentration required for a half-maximal effect was around 100 μ M.

As an alternative approach to determining whether NG108-15 cells contained a functioning Na⁺/Ca²⁺ exchanger, we used the PCR technique. A DNA fragment encoding part of the Na⁺/Ca²⁺

Figure 6 Dependence of ⁴⁵Ca²⁺ uptake on the magnitude of an outwardly directed Na⁺ gradient

NG108-15 cells, without (○) or with (●) ouabain pretreatment, were suspended in Ca²⁺-free Na⁺-free NMG buffer. ⁴⁵Ca²⁺ uptake was initiated by adding cells to test tubes containing 100 μ M ⁴⁵Ca²⁺, 100 μ M verapamil, 10 μ M ω -conotoxin and various concentrations of extracellular Na⁺ as indicated. The required NaCl in the standard buffer was isotonicity replaced with NMG. ⁴⁵Ca²⁺ uptake was terminated after 3 min. Data are means $\pm$ S.D. of one triplicate experiment.

Figure 7 Dependence of the reverse mode of the Na⁺/Ca²⁺ exchanger on extracellular Ca²⁺ concentration

After ouabain pretreatment, NG108-15 cells were suspended in Ca²⁺-free Na⁺-free NMG buffer. ⁴⁵Ca²⁺ uptake was initiated by adding cells to Na⁺-free NMG buffer containing 100 μ M verapamil, 10 μ M ω -conotoxin and various concentrations of Ca²⁺ as indicated. ⁴⁵Ca²⁺ uptake was terminated after 3 min. Data are means $\pm$ S.D. of one triplicate experiment.

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1   CTTAGATGGT GCTCTGGTTC TGGAGGTCGA TGAGAGGGAC CAAGATGACG
51  AGGAAGCCAG GCGTGAGATG GCAAGGATTC TGAAGGAACT TAAGCAGAAG
151 CCTAAGTCAG CAGCAAAAGA GCCGAGCATT TTACCGAATT CAAGCTACTC
201 GCCTGATGAC TGGAGCTGGT AACATTTTGA AGAGGCATGC AGCTGACCAA
251 GCGAGGAAGG CTGTGAGCAT GCATGAAGTC AACATGGATG TGGTTGAAAA
301 TGACCCAGTC AGTAAGGTCT TCTTTGAGCA AGGGACATAC CAGTGCTAG
351 AAAACTGTGG TACTGTGGCC CTCACCATTA TTCGAAGAGG GGGTGACTTG
401 ACCAACACTG TGTGTTGTTGA CTTGAGGACG GAAGATGGCA CAGCCAATGC
451 TGGGTCTGAT TATGAGTTCA CGGAAGGGAC TGTGATTTTC AAACCTGGGG
501 AGACCCAGAA GGAAATCAGA GTTGGCATCA TTGATGATGA TATCTTTGAA
551 GAAGATGAAA ACTTTCTTGT GCATCTTAGC AACGTCAGGG TCTCTTCAGA
601 AGTCTCGGAA GATGGCATACT TAGACTCCAA TCACGTGTCT CGGATTGCTT
651 GTCTCGGGTC ACCCAACT GGCACCATAA CCATTTTGA TGATGACCAC
701 GCGGGCATCT TTACTTTGCA GGAACCCGTG ACTCACGTGA GCGAGAGCAT
751 TGGCATCATG GAGGTGAAGG TGCTGAGAAC CTCAGGAGCT CGAGGAAATG
801 TTATCGTTCC

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Figure 8 Nucleotide sequence of amplified cDNA fragment of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in NG108-15 cells

The numbers of nucleotides are shown on the left.

Table 1 Comparison of nucleotide and deduced amino acid sequences of the amplified cDNA fragment of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in NG108-15 cells with the corresponding fragment from various cell types

Numbers represent the percentage identity in nucleotide and deduced amino acid sequences between the exchanger fragment in NG108-15 cells and that in other cell types as indicated.

Cell	Percentage identity	
	Nucleotide sequence	Deduced amino acid sequence
Rat brain	92.2	99.6
Rat heart	92.1	99.3
Human heart	86.5	94.8
Dog heart	84.8	95.2
Rabbit kidney	77.5	93.3

exchanger was amplified from the first-strand cDNA of NG108-15 cells using primers specific for the largest intracellular loop of the exchanger. The predicted size of the PCR product was 810 bp. To confirm its identity, we subcloned and sequenced this PCR fragment, and the nucleotide sequence is shown in Figure 8. A comparison of nucleotide and deduced amino acid sequences of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in NG108-15 cells with those in cells of various species and tissues including dog heart [15], human heart [14], rat heart [18], rat brain [19] and rabbit kidney [20] is given in Table 1.

DISCUSSION

It has been reported that removal of extracellular Na^+ inhibits the activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which then causes an increase in $[\text{Ca}^{2+}]_i$ in either the basal state or the stimulated state in various cell types, e.g. arterial muscle cells [21], pulmonary

artery endothelial cells [22], chromaffin cells [23,24], platelets [25], synaptosomes [26,27], vascular smooth muscle cells [28] and pancreatic B cells [29]. However, we have demonstrated that, in NG108-15 cells differentiated by dibutyryl cyclic AMP, replacement of extracellular Na^+ does not have any effect on either the basal $[\text{Ca}^{2+}]_i$ or the $[\text{Ca}^{2+}]_i$ transient stimulated by high K^+ or bradykinin (Figure 1). Similar results have been shown previously in platelets stimulated by thrombin [30]. The insensitivity of $[\text{Ca}^{2+}]_i$ change to extracellular Na^+ suggests that the decline in stimulated $[\text{Ca}^{2+}]_i$ is predominantly mediated by Ca^{2+} sequestration within intracellular organelles and the plasma-membrane Ca^{2+} -pumping extrusion system, rather than by the operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Indeed, after exposure of cells to thapsigargin and FCCP to block Ca^{2+} sequestration within intracellular organelles, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger displayed functional importance in the regulation of $[\text{Ca}^{2+}]_i$ (Figures 2–4) even in the absence of agonists. The activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is further shown by Ca^{2+} influx which depends on an outwardly directed Na^+ gradient (Figures 5–7) and is probably mediated by the reverse mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Without ouabain treatment, basal $[\text{Ca}^{2+}]_i$ (●) in Figure 1 and (○) in Figure 6, is not affected by Na^+ replacement. Furthermore, molecular-biology approaches indicate the existence of a $\text{Na}^+/\text{Ca}^{2+}$ exchanger in NG108-15 cells (Figure 8).

We have previously shown using NG108-15 cells that when extracellular Na^+ was replaced with 80 mM K^+ to achieve depolarization, the subsequent rise in $[\text{Ca}^{2+}]_i$ induced by bradykinin was significantly greater than that in control cells not exposed to 80 mM K^+ [31]. We have further demonstrated that the synergistic effect of high K^+ and bradykinin on $[\text{Ca}^{2+}]_i$ was not due to the inhibitory effect of the removal of extracellular Na^+ on the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, and subsequently on Ca^{2+} efflux, because when a 150 mM NMG solution was employed to maintain the tonicity without use of Na^+ , high K^+ still had a similar synergistic effect on the bradykinin-induced increase in $[\text{Ca}^{2+}]_i$. In contrast, in NG108-15 cells, extracellular ATP rapidly raised the $[\text{Ca}^{2+}]_i$; the magnitude of the rise was inversely proportional to the extracellular Na^+ concentration [32,33]. In fact, a non-selective cation channel permeable to Na^+ and Ca^{2+} was activated by ATP. Therefore it would be expected that an enhanced $[\text{Ca}^{2+}]_i$ change would be induced by ATP in the presence of a reduced concentration of Na^+ and would be unrelated to the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Although our present results provide evidence to support the presence of a $\text{Na}^+/\text{Ca}^{2+}$ exchanger in NG108-15 cells, the contribution of the exchanger to the control of $[\text{Ca}^{2+}]_i$ was found to be not very significant unless Ca^{2+} accumulation within intracellular organelles was completely blocked. These results support the notion, proposed by Blaustein et al. [12], that small effects of the exchanger on $[\text{Ca}^{2+}]_i$ are masked by relatively large changes in the amount of Ca^{2+} stored in the endoplasmic reticulum or sarcoplasmic reticulum.

Measurement of the change in $[\text{Ca}^{2+}]_i$ reflects the final $[\text{Ca}^{2+}]_i$ after the operation of all Ca^{2+} transporters within the cell. The insensitivity of $[\text{Ca}^{2+}]_i$ change to extracellular Na^+ (Figure 1) does not necessarily indicate the absence of a $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Indeed, when $^{45}\text{Ca}^{2+}$ uptake driven by an outwardly directed Na^+ gradient was measured, an active reverse mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity was observed (Figures 5–7). Our results suggest that intracellular Ca^{2+} accumulation and Ca^{2+} extrusion via the Ca^{2+} pump may contribute more effectively to the control of $[\text{Ca}^{2+}]_i$ than does the $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

The change in $[\text{Ca}^{2+}]_i$ in response to thapsigargin and FCCP reflected the sum of the $[\text{Ca}^{2+}]_i$ increase caused by Ca^{2+} release from intracellular Ca^{2+} -accumulation organelles and the $[\text{Ca}^{2+}]_i$

decline caused by plasma-membrane Ca²⁺-extrusion mechanisms. Therefore, when extracellular Na⁺ was decreased, the [Ca²⁺]_i increase reached a higher level as a result of the reduced operation of the Na⁺/Ca²⁺ exchanger. After Ca²⁺ was no longer released from intracellular organelles, [Ca²⁺]_i started to decline (Figure 3). Therefore, in the absence of extracellular Na⁺, a slow decay in [Ca²⁺]_i should be observed while thapsigargin and FCCP are present. However, our results (Figure 3a, trace D) demonstrate that Ca²⁺ was still rapidly removed from the cytosol. This may be due in part to the fact that thapsigargin may not be able to block the sequestration of Ca²⁺ totally. A Ca²⁺ pool in which the Ca²⁺ pump is insensitive to thapsigargin exists in DDT₁MF-2 smooth-muscle cells [34]. In addition, we have shown previously that the apparent initial rate of the decline in stimulated [Ca²⁺]_i linearly increases with the [Ca²⁺]_i in intact chromaffin cells [23]. Therefore an enhanced rate of [Ca²⁺]_i decay should be expected at lower concentrations of extracellular Na⁺, in which [Ca²⁺]_i reaches higher levels.

The difference between the transients of [Ca²⁺]_i observed in the presence and absence of extracellular Na⁺ indicates the contribution of the Na⁺/Ca²⁺ exchanger to [Ca²⁺]_i regulation. This difference was more pronounced in cells exposed to thapsigargin and FCCP than in those exposed to thapsigargin alone (Figures 2 and 3). This suggests that dissipating the mitochondrial-membrane potential by using FCCP increases the contribution of the Na⁺/Ca²⁺ exchanger to the lowering of [Ca²⁺]_i. First, the driving force for Ca²⁺ uptake into mitochondria is inhibited by FCCP. Second, FCCP blocks the production of ATP and thereby reduces its concentration. Consequently, Ca²⁺ extrusion via the Ca²⁺ pump is inhibited. Therefore, after exposure of cells to thapsigargin and FCCP, the Na⁺/Ca²⁺ exchanger becomes important in the regulation of [Ca²⁺]_i. On the other hand, our results indirectly indicate that mitochondria are functionally important in controlling [Ca²⁺]_i under physiological conditions in NG108-15 cells. In contrast, it has been shown previously that, in synaptosomes, FCCP causes a slight and identical increase in [Ca²⁺]_i, no matter whether synaptosomes are suspended in a normal- or a low-Na⁺ (24–36 mM) medium [26], indicating that little of the Ca²⁺ that is taken up by the synaptosomes in the low-Na⁺ medium is sequestered by the mitochondria. It has been established that mitochondria possess a large capacity to accumulate Ca²⁺, but they have only limited effect on [Ca²⁺]_i because of their low affinity for Ca²⁺ [7,35–37].

It has been shown previously that, in synaptosomes, FCCP has no effect on the ⁴⁵Ca²⁺ uptake induced by high K⁺ when extracellular Ca²⁺ is low (10 or 20 μM) and K⁺-stimulated ⁴⁵Ca²⁺ uptake is small. However, with a higher extracellular Ca²⁺ concentration (200 μM) and larger high-K⁺-stimulated Ca²⁺ uptake, FCCP reduces Ca²⁺ uptake by about one-third [27]. Thus a substantial amount of ⁴⁵Ca²⁺ is taken up by mitochondria when the extracellular ⁴⁵Ca²⁺ concentration is high. In order to prevent the large accumulation of ⁴⁵Ca²⁺ within mitochondria from masking the measurement of the activity of the Na⁺/Ca²⁺ exchanger, we used a lower concentration of Ca²⁺ (100 μM) when ⁴⁵Ca²⁺-uptake experiments were undertaken in the present study. A reverse mode of the Na⁺/Ca²⁺ exchanger activity was demonstrated in NG108-15 cells no matter how the Ca²⁺ influx was determined, by using either the isotope ⁴⁵Ca²⁺ (Figures 6 and 7) or the Ca²⁺ fluorescence indicator fura 2 (Figure 5). The activity of the reverse-mode exchanger was dependent on the magnitude of the outwardly directed Na⁺ gradient (Figures 5 and 6) and extracellular Ca²⁺ concentration (Figure 7).

In previous studies, we found that undifferentiated NG108-15 cells did not exhibit a significant depolarization-evoked increase in [Ca²⁺]_i; however, depolarization did induce a greater rise in

[Ca²⁺]_i after cells had been differentiated by elevating the cellular cyclic AMP level [38]. In the current study, dibutyryl cyclic AMP-differentiated NG108-15 were used and voltage-operated Ca²⁺ influx caused by cytosolic Na⁺ elevation was inhibited by the Ca²⁺-channel blockers, verapamil and ω-conotoxin. It is possible that there may still be a voltage-operated Ca²⁺ influx through blocker-insensitive channels. Hence it would be expected that increasing the extracellular Na⁺ concentration would reduce the potential across the membrane and so reduce blocker-insensitive channel-mediated Ca²⁺ entry. In fact, in a previous study [38] we showed that nifedipine and ω-conotoxin could almost completely block the depolarization (80 mM K⁺)-induced [Ca²⁺]_i increase (by approx. 96%). Therefore, in the present study, the enhanced [Ca²⁺]_i increase observed in the presence of a reduced concentration of extracellular Na⁺ reflected the activity of a Na⁺/Ca²⁺ exchanger.

The deduced amino acid sequence of the amplified DNA fragment of the Na⁺/Ca²⁺ exchanger from NG108-15 cells differs by a single amino acid residue from the sequence of the exchanger in rat brain [19]; this residue is valine in NG108-15 cells and isoleucine (residue 569) in rat brain. The identities in the nucleotide and amino acid sequences are 92.2% and 99.6% respectively (Table 1).

In conclusion, the present evidence suggests the presence of a Na⁺/Ca²⁺ exchanger in NG108-15 cells.

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